PATENT APPLICATION
ATTORNEY DOCKET NO. 27866/32663

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	)	For: METHODS FOR USING AGENTS THAT BIND TO VCAM-1 (Amended Title)
Masinovsky et al.	)	
Serial No: 08/448,649	)	Group Art Unit: 1815
Filed: May 24, 1995	)	Examiner: P. Gambel, Ph.D.

SECOND DECLARATION OF THALIA PAPAYANNOPOULOU, M.D., Dr. Sci., UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, Thalia Papayannopoulou, M.D., Dr. Sci., hereby declare and say as follows:

1. I received my M.D. and my Dr. Sci. from the University of Athens in 1961 and 1964, respectively. I completed my internship and Assistantship in Medicine and Hematology in Greece. I was an NIH Fellow and Senior Fellow in the Department of Hematology at the University of Washington, Seattle, Washington. Since 1978, I have been on the faculty of the Department of Medicine of the University of Washington. I have been a Professor in the Division of Hematology since 1984 and Director of the Hematology Training Program since 1990. I have been on numerous committees of the American Society of Hematology and the International Society of Experimental Hematology. I was awarded the William

Dameshek Prize by the American Society of Hematology in 1990. I have authored or co-authored at least 215 publications. I have done extensive research into hematopoietic stem cells, including peripheralization of hematopoietic stem cells.

2. I have reviewed the above-identified application and its currently pending claims, and I have been informed that the Examiner has rejected the claims on the ground that one of ordinary skill in the art would not be able to carry out the claimed methods. I believe I am qualified by education and training to comment on what one of ordinary skill in the art relating to bone marrow cells would have been able to accomplish as of August 2, 1990, which I have been advised is the effective filing date of the above-identified application.

3. I make the following statements to respond to the Examiner's position that:

> The disclosure does not provide direction or guidance as to which therapeutic conditions and what therapeutic endpoints are would be [sic] appropriate for the claimed methods.
> . . . The specification does not teach how to extrapolate data obtained from in vitro binding studies of marrow stromal elements to the development of effective in vivo therapeutic methods to interfere with hemopoietic cell-marrow stroma interactions, commensurate in scope with the claimed invention. Undue experimentation would be required to practice the claimed methods with a reasonable expectation of success . . .

4. I disagree with the Examiner's position. The Applicants have claimed a method of blocking interaction between a bone marrow stromal cell expressing VCAM-1 and a hemopoietic precursor cell by administering an anti-VCAM-1 antibody in an amount effective to decrease VCAM-1-mediated adhesion between the bone marrow stromal cell and the hemopoietic cell. The application teaches that an anti-VCAM-1 antibody, 6G10, binds to bone marrow stromal cells in *in vitro* culture, and that VCAM-1 is implicated in the binding of bone marrow stromal cells to hemopoietic cells. In light of the knowledge conveyed by the application, one of ordinary skill in the art as of August 2, 1990 would readily have been able to administer an amount of anti-VCAM-1 antibody effective to achieve the therapeutic endpoint, which is the decrease of VCAM-1-mediated adhesion between stroma and hemopoietic precursors, resulting in mobilization of hemopoietic cells. It would have required no more than routine experimentation for the ordinary skilled worker to determine what therapeutic conditions would provide a desired level of measured response.

5. Data reported in my previous declaration signed November 30, 1995 confirm that one of ordinary skill in the art could readily have practiced the claimed methods *in vivo*. Antibody 6G10 was administered to two non-human primates using two different dosage regimens. One animal received a single 2 mg/kg (body weight) dose of antibody intravenously, while the other received a 2 mg/kg dose of antibody intravenously on day 0, on day 1 and on day 2. The data reported

in my previous declaration demonstrated that under both treatment regimens, systemic administration of antibody 6G10 caused release of bone marrow progenitor cells from the bone marrow to the peripheral blood. No undue experimentation was involved in order to achieve this therapeutic endpoint.

6. Data reported in Papayannopoulou *et al., Proc. Nat'l. Acad. Sci (USA), 92*:9647-9651 (1995) (Exhibit 1), further confirm that one of ordinary skill in the art could readily have practiced the claimed methods *in vivo* with a different anti-VCAM-1 antibody, MK/2. Antibody MK/2 was intravenously administered to mice at a dosage of 2 mg/kg (body weight) daily for three days. Cells in the peripheral blood were assayed for granulocyte/macrophage colony forming units (CFU-GM) and erythroid burst-forming units (BFU-e), and the numbers were pooled for reporting as culture colony forming units (CFU-C). Treatment with anti-VCAM-1 antibody resulted in 361 $\pm$ 42.3 CFU-C/ml blood, compared to 119 $\pm$ 10.6 CFU-C/ml blood for mice treated with an isotype control antibody ($p < 0.01$). See page 9650, first column. These results demonstrate that systemic administration of antibody MK/2 caused release of bone marrow progenitor cells from the bone marrow to the peripheral blood. No undue experimentation was involved in order to achieve this therapeutic endpoint.

7. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

August 19th 1997
Date

Thalia Papayannopoulou, M.D., Dr. Sci.

Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9647–9651, October 1995
Cell Biology

The VLA_4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen

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ABSTRACT **Selective lodgement or homing of transplanted hemopoietic stem cells in the recipient's bone marrow (BM) is a critical step in the establishment of long-term hemopoiesis after BM transplantation. However, despite its biologic and clinical significance, little is understood about the process of homing. In the present study, we have concentrated on the initial stages of homing and explored the functional role *in vivo* of some of the adhesion pathways previously found to mediate *in vitro* adhesion of hemopoietic cells to cultured BM stroma. We have found that homing of murine hemopoietic progenitors of the BM of lethally irradiated recipients at 3 h after transplant was significantly reduced after pretreatment of the donor cells with an antibody to the integrin very late antigen 4 (VLA_4). This inhibition of marrow homing was accompanied by an increase in hemopoietic progenitors circulating in the blood and an increased uptake of these progenitors by the spleen. Similar results were obtained by treatment of the recipients with an antibody to vascular cell adhesion molecule 1 (VCAM-1), a ligand for VLA_4. Furthermore, we showed that administration of the same antibodies (anti-VLA_4 or anti-VCAM-1) to normal animals causes mobilization of hemopoietic progenitors into blood. These data suggest that hemopoietic cell lodgement in the BM is a regulatable process and can be influenced by VLA_4/VCAM-1 adhesion pathway. Although additional molecular pathways are not excluded and may be likely, our data establish VCAM-1 as a BM endothelial addressin, analogous to the role that mucosal addressin cell adhesion molecule (MAdCAM) plays in lymphocyte homing. Whether splenic uptake of hemopoietic progenitors is passive or controlled through different mechanisms remains to be clarified. In addition, we provide experimental evidence that homing and mobilization are related phenomena involving, at least partly, similar molecular pathways.**

Normal hematopoiesis in the adult is restricted within bone marrow (BM) spaces where hemopoietic cells are found in intimate association with BM stromal cells and the extracellular matrix, which make up the hemopoietic microenvironment. Adhesive interactions of hemopoietic cells with BM stroma are thought to critically influence their self-renewal, differentiation, and release into the blood stream. Furthermore, the establishment of long-term hematopoiesis after transplantation is critically dependent on the ability of transplanted hemopoietic cells to home to the BM where they rapidly proliferate and differentiate (1–5). However, the steps that lead to the process referred to as homing have not been defined, and the molecular pathways that control these steps have not been elucidated.

Previous studies on BM engraftment *in vivo* have drawn attention to the importance of lectins on hemopoietic cells and glycoconjugates on BM stroma (2–5). Neither the nature of putative hemopoietic progenitor lectins nor the glycoconjugate ligands on BM have been precisely defined. In addition, other studies have suggested that medullary and splenic engraftment is compromised when transplanted cells were treated with polyclonal antibodies to β_1, the common subunit of very late antigen (VLA) integrins (6). *In vitro* studies have also concluded that adhesive interactions between hemopoietic cells and their BM stroma, mediated through several cytoadhesion pathways, are responsible for the anchoring of hemopoietic cells in BM (7–15). However, cultured BM stromal cells were used, and these may not faithfully reproduce the complex hemopoietic microenvironment *in vivo* (16). Moreover, the relevance of these *in vitro* studies to the *in vivo* BM recognition, transmigration, and firm attachment of transplanted hemopoietic cells to BM stroma has not been explored. In contrast, *in vivo* studies on homing of lymphocytes to peripheral lymph nodes or to mucosal tissues have provided valuable insights (17–19). Likewise, the cascade of adhesive interactions between neutrophils and endothelial cells has been studied in detail and the molecular pathways have been delineated (20). In contrast to these studies, however, *in vivo* studies concerning cytoadhesive interactions of hemopoietic progenitor cells have lagged behind.

In the present study, we have explored the parameters that influence the initial stages of homing of transplanted hemopoietic progenitors within the BM. By treating donor cells with anti-VLA_4 or recipients with anti-vascular cell adhesion molecule 1 (VCAM-1), we showed that the VLA_4/VCAM-1 adhesion pathway plays a role in the initial stages of homing within the BM. Since splenic homing was not affected in these experiments, and in fact was increased, the data suggest operationally distinct pathways controlling BM and splenic homing. Finally, as antibody treatment of normal animals with anti-VLA_4 or VCAM-1 induces mobilization of hemopoietic progenitors into circulation, a putative molecular link between hemopoietic progenitor homing and mobilization is suggested.

MATERIALS AND METHODS

Mice, Irradiation, and Cell Harvest. Specific pathogen-free $(C57BL/6 \times DBA_2)F_1$ ($B_6D_2F_1$) mice 3–6 months old were used in all experiments. Mice were allowed acidified water and food ad libitum and maintained in a filtered-air-flow housing unit for the duration of the experiment. Mice used as primary or secondary recipients in the homing assay were exposed to 1150-cGy irradiation delivered from a Cs source as described (21).

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Abbreviations: VLA, very late antigen; VCAM-1, vascular cell adhesion molecule 1; BM, bone marrow; CFU-C, CFU-GM, and CFU-S, culture, granulocyte/macrophage, and spleen colony-forming unit(s), respectively; BFU-e, burst-forming unit(s), erythroid.

Single cell suspensions were prepared from peripheral blood, BM, and spleen. Peripheral blood was obtained in preservative-free heparin by cardiac puncture and nucleated cells were recovered using NH_4Cl hemolytic buffer. BM cells were obtained by flushing femoral marrow, under sterile conditions, with Iscove's modified Dulbecco's medium (IMDM) containing 10% (vol/vol) fetal bovine serum (Summit Biotechnology, Ft. Collins, CO) and streptomycin (50 μg/ml) and penicillin (50 units/ml) (Irvine Scientific). Splenic cell suspensions were obtained by lengthwise dissection of the spleen with a scalpel and scraping of cellular contents from the capsule, followed by vigorous pipetting. All cell suspensions were washed twice in IMDM with 10% fetal bovine serum. Cell counts were obtained by using a hemocytometer.

Culture Colony-Forming Units (CFU-C) Assay. CFU-C assays were performed by using a methylcellulose mixture consisting of 1.2% (wt/vol) methylcellulose (Fisher Scientific), 30% fetal bovine serum (HyClone), 1% bovine serum albumin, 0.1 mM 2-mercaptoethanol (Eastman Kodak), recombinant erythropoietin (Genetics Institute, Cambridge, MA; 5 units/ml), 10% (vol/vol) WEHI-3 conditioned medium, 5% (vol/vol) pokeweed-mitogen-stimulated spleen cell conditioned medium, and recombinant rat stem cell factor (Amgen Biologicals; 50 ng/ml), in IMDM. Cultures were set up in duplicate and maintained in a high humidity 37°C in a 5% CO_2/95% air incubator. BM and spleen cells from transplanted primary recipients were plated in methylcellulose cultures at 5×10^5 and 1×10^6 cells per ml, respectively. The cells from 0.25–0.5 ml of red-cell-lysed peripheral blood were plated in 1 ml of methylcellulose mixture. After 10–12 days granulocyte/macrophage colony-forming units (CFU-GM) and burst-forming units, erythroid (BFU-e), colonies were counted on the basis of morphological criteria under a dissecting microscope. CFU-GM and BFU-e numbers were pooled and reported as CFU-C. Donor BM cells were plated at a concentration of 5×10^4 cells per ml to determine CFU content of marrow inoculum used for transplantation.

Spleen Colony-Forming Units ($CFU\text{-}S_{11}$) Assay. CFU-S day 11 were assessed by injecting an appropriate aliquot of donor BM mononuclear cells into the tail vein of a lethally irradiated recipient mouse. Mice were sacrificed 11 days later, and their spleens were excised and suspended in Bouin's medium, followed by 10% (vol/vol) neutral buffered formalin. The numbers of macroscopically visible surface colonies (CFU-S) were counted by the same observer for all experiments. In selected experiments, mice were sacrificed at both days 8 and 11 after transplant.

Antibodies to VLA_4 and VCAM-1. Endotoxin-free PS/2, a rat IgG2a antibody that recognizes the α4 chain of murine VLA_4, was used as an anti-VLA_4 antibody (7). MK/2, a rat IgG1 antibody that recognizes murine VCAM-1, was used (8) as an anti-VCAM-1 antibody. Isotype-matched IgG material was given to control animals.

Assessment of Hemopoietic Progenitor Homing and Mobilization. Homing of CFU-S and CFU-C to BM or spleen was assessed 3 h after the i.v. injection of donor cells into primary recipients, based on data from control animals in which transplanted cells are almost completely cleared from the circulation by this time (22). To measure CFU-S content, we retransplanted an appropriate inoculum of BM (i.e., 25% of a femur) or spleen cells (i.e., 2.5% of a spleen) into secondary recipient groups (at least 10–15 mice per test sample). These secondary groups were sacrificed at day 8 or 11 and macroscopic spleen colonies representing the $CFU\text{-}S_8$ and $CFU\text{-}S_{11}$ content of the test sample were counted. In experiments with anti-VLA_4, donor BM cells were incubated with the antibody or isotype controls for 40 min prior to injection. In the studies with anti-VCAM-1, the antibody or isotype control was given i.v. [2 mg/kg (body weight)] to irradiated recipients 1 h before transplantation of normal donor BM cells.

To measure CFU-C lodged in the BM or spleen of primary recipients and those present in the blood 3 h after transplantation, we used BM, spleen, and blood samples from the same primary recipients as for CFU-S assays to inoculate *in vitro* semisolid cultures. By comparing the numbers of CFU-C present in each tissue (BM, spleen, and blood) to the total CFU-C transplanted (determined by CFU-C assay of donor cell inoculum), we can also calculate the recovery of CFU-C (as percent of injected) in each of these tissues in control and antibody-treated animals.

To assess hemopoietic progenitor mobilization in normal animals, we administered the antibodies, either anti-VLA_4 or anti-VCAM-1, intravenously for 3 days (as a single dose per day) and antibody-treated mice and isotype-treated controls were sacrificed on the fourth day. Nucleated cells present in 0.25–0.5 ml of peripheral blood were plated in clonogenic progenitor cultures to assess CFU-C content, as described above.

Statistics. A Student's two-tailed *t* test was used to uncover significance of differences in paired results.

RESULTS

Preincubation with an Antibody to Murine VLA_4 Decreases Homing of $CFU\text{-}S_{11}$ and CFU-C to BM. We examined the role of VLA_4 in hemopoietic progenitor homing by comparing the distribution of hemopoietic progenitors in irradiated mice 3 h after the transplantation of normal donor BM cells incubated with either anti-murine VLA_4 or an isotype-matched control (IgG2a). Incubation with anti-VLA_4 resulted in a 48% reduction in the number of $CFU\text{-}S_{11}$ homing to the femurs of primary recipient mice (assessed in 10 secondary recipients) compared with an isotype control (Fig. 1*A*, $P < 0.001$). In contrast to its effect on marrow homing, preincubation with anti-VLA_4 resulted in a 139% increase in $CFU\text{-}S_{11}$ in the spleen of primary recipients compared to controls 3 h after transplantation (Fig. 1*A*, $P < 0.0005$). No difference was noted in the overall cellularity of either femurs or spleens between control and treated primary recipients.

When CFU-C were used as an assay of hemopoietic progenitors, anti-VLA_4 produced a similar alteration in marrow (29% of control) and splenic (124% of control) homing as observed for $CFU\text{-}S_{11}$ (Fig. 2*A*), 3 h after transplantation. Increased numbers of CFU-C were also present in peripheral blood of mice transplanted with anti-VLA_4-treated compared to isotype-control-treated cells (Fig. 2*A*, $P < 0.0003$).

Administration of an Antibody to Murine VCAM-1 Decreases the Homing of $CFU\text{-}S_{11}$ and CFU-C to BM. We next studied hemopoietic progenitor homing in the presence of a rat anti-murine VCAM-1 antibody. In view of the predominant expression of VCAM-1 on endothelium and BM stroma (8), homing was studied in irradiated recipient mice treated with intravenous anti-VCAM-1 2 h prior to transplantation. Such pretreatment produced a 54% reduction in primary marrow $CFU\text{-}S_{11}$ collected 3 h after transplantation (Fig. 1*B*, $P < 0.004$). In addition, pretreatment with MK/2 increased the numbers of splenic $CFU\text{-}S_{11}$ compared with isotype controls (Fig. 1*B*, $P < 0.005$). A second experiment yielded similar results (data from both experiments presented in Fig. 1*B*, $P < 0.0002$ for BM and $P < 0.0003$ for spleen). When hemopoietic progenitors were assayed as CFU-C, changes in homing to BM and spleen were similar to those observed with CFU-S (Fig. 2*B*). As with anti-VLA_4-treated cells, increased numbers of CFU-C were present in the peripheral blood of anti-VCAM-1-treated mice (783% of control) 3 h after transplantation (Fig. 2*B*, $P < 0.02$).

Simultaneous Administration of Anti-VLA_4 and Anti-VCAM-1 Reduces the Homing of $CFU\text{-}S_{11}$ and CFU-C to BM. To test whether treatment with both anti-VLA_4 and anti-VCAM-1 exerted additive effects on hemopoietic progenitor

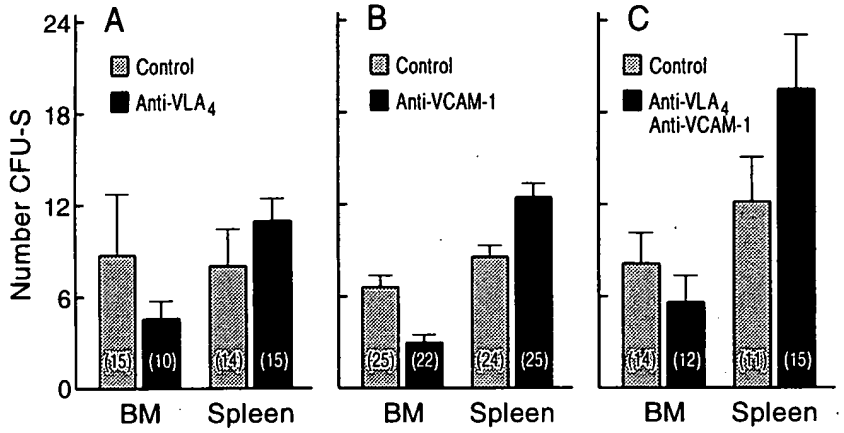

FIG. 1. CFU-S_{11} counted in spleens of secondary irradiated recipients that received samples from BM or spleen of primary irradiated, control, or antibody-treated recipients. Number of CFU-S in spleens of secondary recipients reflects the CFU-S lodged in BM or spleen of primary recipients 3 h after transplantation. (Numbers in parentheses in each column indicate the number of secondary recipients used. In *B*, the data from two experiments are pooled.) Note consistent reduction in CFU-S lodged in BM in animals treated with anti-VLA_4 (*A*), anti-VCAM-1 (*B*), or both anti-VLA_4 and anti-VCAM-1 (*C*), in contrast to increase in splenic lodgement of CFU-S.

homing, we transplanted anti-VLA_4-treated donor cells into anti-VCAM-1-treated recipient mice. Combined treatment with both antibodies resulted in a 30% reduction in hemopoietic progenitor homing to BM compared with isotype controls (Fig. 1*C*, $P < 0.002$). The content of CFU-C in the anti-VLA_4 and anti-VCAM-1 primary recipient mice (three mice) was also studied and compared to four controls (treated with IgG2a and IgG1 matched isotypes). A reduction in BM (≈30%) and an increase in splenic (190%) homing were again noted (Fig. 2*C*). In addition, more than twice the number of CFU-C was found in the peripheral blood of the anti-VLA_4- and anti-VCAM-1-treated mice (Fig. 2*C*, $P < 0.004$). Thus, simultaneous administration of anti-VLA_4 and anti-VCAM-1 antibodies causes a reduction in hemopoietic progenitor homing to the BM that is accompanied by increases in the numbers of hemopoietic progenitors in the spleen and peripheral blood comparable to that seen when the antibodies are administered separately.

The Reduction in Hemopoietic Progenitor Homing Associated with Anti-VLA_4 and Anti-VCAM-1 Administration Is Maintained 40 h After Transplantation. Since hemopoietic progenitors may continue to circulate after transplantation, we studied whether the effect of anti-VLA_4 and anti-VCAM-1 treatment was maintained for >3 h after transplantation. Our data show that the reduction in progenitor homing to the BM noted 3 h after transplant was maintained 40 h later (Fig. 3, $P < 0.0001$). A similar reduction was observed when hemopoietic progenitors were assayed by using CFU-C as an assay. It is of interest that although CFU-C were still reduced in treated compared to control primary recipients at 40 h after transplantation, this was not due to a failure of proliferation of antibody-treated cells, since, as seen in Fig. 3, the fraction of hemopoietic progenitors lodged in BM (as percent of injected CFU-C) increased in both groups.

Administration of Anti-VLA_4 or Anti-VCAM-1 Causes Hemopoietic Progenitor Mobilization. The decrease in hemopoietic progenitor homing caused by administration of anti-VLA_4 or anti-VCAM-1 to irradiated recipients was associated with a simultaneous increase in their numbers in peripheral blood. Therefore, we wished to study whether these antibodies were able to mobilize hemopoietic progenitors into the blood of normal unirradiated mice. In these animals, administration of three daily injections of anti-VLA_4 was associated with a >16-fold increase in the numbers of CFU-C in the peripheral

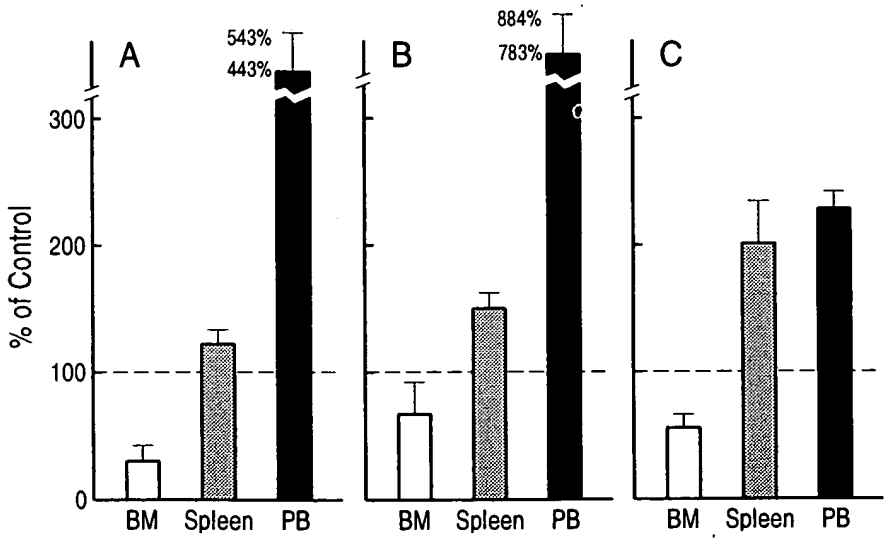

FIG. 2. CFU-C present per organ [femur, spleen, and 1 ml of blood (PB)] in irradiated recipients 3 h after injection of donor BM cells. CFU-C were assessed by *in vitro* clonogenic assays and are expressed as percent of control values (mean ± SEM). (*A*) Anti-VLA_4-treated group. (*B*) Anti-VCAM-1-treated group. (*C*) Anti-VLA_4- and anti-VCAM-1-treated group. Note the significant increase in blood CFU-C present at 3 h after transplantation and the increase in the spleen in all treatment groups, compared to a decrease in BM.

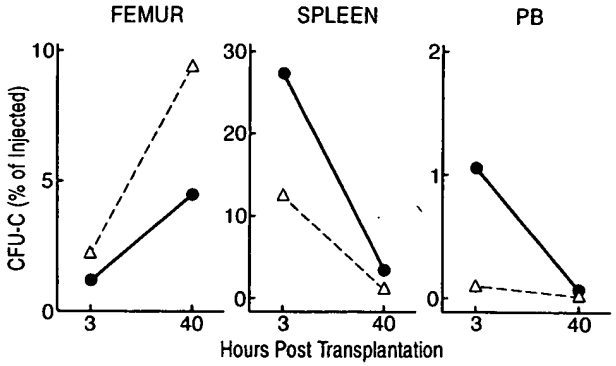

FIG. 3. CFU-C present in femur, spleen, and peripheral blood (PB) of primary irradiated recipients. Control (△) and treated with both anti-VCAM-1 and anti-VLA$_4$ (●) are shown at 3 and 40 h after transplantation of donor BM cells. CFU-C assessed in *in vitro* clonogenic assays by plating an appropriate inoculum from these three cell sources. The total number of CFU-C injected was also estimated by clonogenic assay of the donor BM inoculum. The data are presented as proportion of injected CFU-C present in each organ at 3 and 40 h after transplantation. Note that in BM, the proportion of CFU-C increased between 3 and 40 h (due to cell proliferation), but differences between control and treated animals were maintained. A decrease in spleen CFU-C at 40 h has been observed in previous studies (25, 26) and attributed to anatomic reasons (vascular collapse after irradiation) (30). Also note that at 40 h very few CFU-C are present in circulation.

blood compared with the effect of administration of an isotype control (Fig. 4, $P < 0.001$). Both CFU-GM and BFU-e were mobilized; however, late maturational stages (hemopoietic precursor cells) were not, in agreement with our previous data in primates (23). Administration of anti-VCAM-1 using a similar schedule (three i.v. injections) was also associated with a significant hemopoietic progenitor mobilization although the effect was less marked than that of anti-VLA$_4$ (isotype control, 119 ± 10.6 CFU-C per ml of blood; anti-VCAM-1 treated, 361 ± 42.3 CFU-C per ml; $P < 0.01$).

DISCUSSION

This study, demonstrating that antibodies to VLA$_4$ and VCAM-1 inhibit hemopoietic progenitor homing to BM, implicates the participation of the cognate cytoadhesion molecules in the initial stages of recognition of transplanted hemopoietic cells by the BM sinusoidal endothelium. We

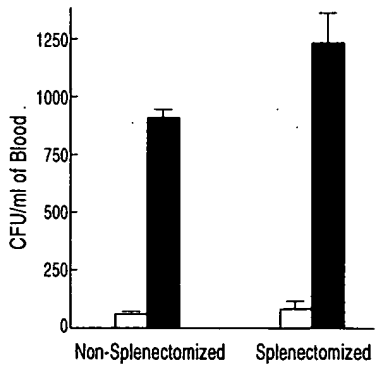

FIG. 4. Total CFU-C per ml of peripheral blood present in two groups of animals, one treated with three intravenous injections (one injection per day for 3 days) of isotype control antibody (open bars) and the other treated with anti-VLA$_4$ (solid bars). Both normal and splenectomized mice were treated this way and all groups were sacrificed on day 4 (day after the last treatment) for testing. Note significant increases of CFU in peripheral blood of anti-VLA$_4$-treated animals (solid bars) compared to controls (open bars).

interpret the effect observed with both anti-VLA$_4$ and anti-VCAM-1 as a consequence of the blockade of the VLA$_4$/VCAM-1 adhesion pathway. However, it is unclear at this stage whether the inhibition of BM homing was caused by a decreased adherence of transplanted hemopoietic progenitors to the luminal surface of BM endothelium or by impairing, in addition, their transmigration through the endothelium or subsequent fixation within the BM stroma.

The decreased homing of progenitor cells to the BM was accompanied by an increase in the numbers of progenitors circulating in the blood and an increase of those present in the spleen of antibody-treated animals. The data thus argue for a redistribution from the BM to circulation and thence to the spleen rather than a selective loss or destruction of progenitor cells within the BM. The increase in the spleen is considered as consequent to the increased pool of circulating progenitors and the antibody's lack of interference with the spleen's ability to capture these cells. These data further suggest that in the murine model splenic homing is mediated by different molecular pathways than those operating in BM homing. As the red pulp of the spleen, being unconstrained by a rigid cortex like that of BM, is more expandable to the circulating pools of cells, it is also possible that splenic uptake is passive and dictated only by anatomic reasons. Alternatively, differences in the functional status of VCAM-1 expressed by endothelial sinusoids of the BM compared to spleen may underlie the selectivity of BM homing. This concept is compatible with recent work suggesting that VCAM-1 is constitutively expressed only in BM (8, 13). Apart from the spleen, it is unlikely that circulating cells lodged in large numbers in other nonhemopoietic organs, as this would have obscured the increase in circulating progenitors.

Since antibodies to VCAM-1 produced a similar effect to those against VLA$_4$, one may suggest that VCAM-1 is the significant physiological ligand for VLA$_4$ present in hemopoietic progenitors. Combined use of the two antibodies did not decrease homing to a greater degree than either used alone, suggesting that either one alone produced a maximal blockade of this receptor–ligand event. As all treatments did not completely abolish BM homing, other cytoadhesion molecules expressed on hemopoietic progenitors and their counter receptors in stromal cells may cooperate to secure their homing and stabilization within the BM *in vivo*. For example, the contribution of the other dominant ligand for VLA$_4$, the CS-1 moiety of fibronectin (24), awaits further study. It is of note, however, that the anti-VLA$_4$ antibody used in our studies blocks both VCAM-1- and CS-1-dependent adhesion *in vitro* (7). Furthermore, both VCAM-1 and CS-1 serve as ligands, not only for VLA$_4$ but also for the $\alpha_4\beta_7$ integrin (LPAM-1), responsible for lymphocyte homing to mucosal tissues, through interaction with its high-affinity ligand mucosal addressin cell adhesion molecule (MAdCAM) (18). Presence of $\alpha_4\beta_7$ in hemopoietic cells, however, has not been explored. Lectin-like molecules on hemopoietic cells and glucoconjugates on BM stroma may also play a role.

Several previous studies have shown that hemopoietic progenitors are found in many tissues (lung, liver, kidney, BM, and spleen) during the first few hours after cell infusion in irradiated recipients (25–27). Collectively, these early studies and experiments in our laboratory (data not shown) suggest no preferential uptake of hemopoietic cells by BM or spleen. Their presence, however, in nonhemopoietic tissues is transient, so that by 48 h the only tissues harboring hemopoietic progenitors are BM and spleen. Whether the cells are preferentially retained in hemopoietic tissues once they land there or just preferentially survive and proliferate in these tissues was not clear. The data presented here showing a selective modulation of BM lodgement 3 h after cell infusion and before any proliferative influence has taken place favor a preferential

retainment/survival of hemopoietic progenitors once they encounter the BM.

Modulation of the *in vivo* "homing" of transplanted hemopoietic progenitors has been reported on two occasions (5, 6). However, homing, as defined by the immediate process of the lodgement of circulating progenitors, was not directly measured in either of these two studies but was inferred from alterations in marrow and spleen cellularity 8–12 days after transplantation. This experimental approach does not make it possible to distinguish effects on hemopoietic progenitor uptake from alterations in subsequent rates of cellular proliferation. Moreover, in contrast to our findings, the importance of VLA_4/CS-1 pathway for both BM and splenic engraftment was stressed in one of the studies (6), whereas the other (5) synthetic neoglycoproteins interacting with putative lectins on hemopoietic progenitors were found to influence BM homing, but only in splenectomized animals (5). Neither of these studies examined the circulation of hemopoietic progenitors in the peripheral blood after transplantation. Furthermore, it needs to be emphasized that all prior studies examined hemopoietic progenitors (CFU-S and CFU-C), as we did, and not the long-term repopulating cells, which may theoretically display a different homing behavior.

In steady-state hemopoiesis, a small proportion of hemopoietic progenitors circulate in the peripheral blood, but these can be increased to significant numbers by treatment with cytotoxic drugs or with various cytokines (28). The mechanism(s) by which progenitors are mobilized remains elusive. Our findings (present paper and ref. 23) that the same antibodies that decrease hemopoietic progenitor homing to the BM also cause their mobilization in normal animals have the following implications. (*i*) They suggest that VCAM-1 inhibition by antibody treatment occurs not only on the irradiated endothelium but also on the normal unstimulated BM endothelium expressing a functional VCAM-1. (*ii*) They provide experimental evidence that similar molecular pathways may be involved in homing and mobilization. As hemopoietic precursors of intermediate maturity were not released by these treatments, the data also imply that homing and mobilization are separately regulated from the release of maturing hemopoietic cells to the periphery. By additional experiments in a xenogeneic model, we have also shown that the mechanism of homing and mobilization, implemented in part through VLA_4/VCAM-1 pathway, is well conserved across species (29). Thus, inhibition of VLA_4/VCAM-1 pathway may have a clinical utility in boosting collections of peripheral blood stem cells used for transplantation.

In summary, our results suggest a molecular pathway by which, at least in part, both homing and mobilization of hemopoietic progenitors can be modulated and define a strategy to examine the role of other cytoadhesion molecules in both hemopoietic progenitor homing and trafficking.

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